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Involvement of the AAA ATPase p97/VCP in DNA metabolism

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1. Summary / Zusammenfassung

1.1 English

Cell cycle checkpoints are essential in eukaryotic cells to maintain the integrity of the genome despite constant exposure to endogenous and exogenous stressors attacking the DNA molecule. The aim of this study was to characterize the influence of the AAA ATPase p97/VCP on the functional efficiency of the G2/M cell cycle checkpoint. Since the specificity of p97/VCP is given by interaction with distinct adaptors, the Npl4 subunit of the heterodimeric main adaptor Ufd1/Npl4 was also part of the project. Additionally, smc5 and mms21 were investigated in this context as they are supposed to be potential substrates of p97/VCP. For this purpose, the proteins of choice were down regulated in U2OS cells by using siRNA. Mitotic indices of control cells and cells exposed to ionizing radiation were determined to monitor the activity of the checkpoint. This was performed in cells randomly distributed throughout the cell cycle as well as in synchronized cells. The results indicated that the G2/M checkpoint is neither significantly affected by p97/VCP nor by Npl4, smc5 or mms21. However, these results gave reason to investigate the role of p97/VCP, Npl4 and mms21 in DNA replication. Surprisingly, FACS cell cycle analysis then revealed a delayed onset of the S phase in cells depleted for p97/VCP and Npl4.

1.2 Deutsch

Kontrollpunkte im Zellzyklus eukaryotischer Zellen sind unentbehrlich um die Integrität des Genoms, trotz fortwährenden Attacken endogener und exogener Stressoren auf die DNA, sicher zu stellen. Das Ziel dieser Arbeit war es, den Einfluss der AAA ATPase p97/VCP auf die Funktionstüchtigkeit des G2/M Kontrollpunktes zu charakterisieren. Da die Spezifität von p97/VCP auf der Interaktion mit bestimmten Adaptoren basiert, war Npl4, eine Untereinheit des heterodimeren Hauptadaptors Ufd1/Npl4, ebenfalls Teil des Projektes. Zusätzlich wurde der Einfluss von smc5 und mms21, zwei potentielle Substrate von p97/VCP, genauer untersucht. Zu diesem Zweck wurden U2OS Zellen mit siRNA behandelt, um das gewünschte Protein vorübergehend auszuschalten. Zur darauf folgenden Überwachung der Aktivität des Kontrollpunktes wurde der mitotische Index bestimmt. Dies wurde sowohl in Kontrollzellen durchgeführt, wie auch in Zellen, welche mit 3 Gy respektive 10 Gy bestrahlt wurden. Die erhaltenen Resultate zeigten, dass in unsynchronisierten, wie auch in synchronisierten Zellen der G2/M Kontrollpunkt durch p97/VCP, Npl4, smc5 und mms21 nicht wesentlich beeinflusst wird. Jedoch haben dieselben Resultate Anlass dazu gegeben, p97/VCP, Npl4 und mms21 im Zusammenhang mit der DNA Replikation zu untersuchen. Wenn Zellen mit siRNA gegen p97/VCP und Npl4 behandelt wurden, zeigte die Zellzyklusanalyse mittels FACS einen verzögerten Eintritt in die S Phase.

2. Introduction

2.1 Cell cycle checkpoints

Over millions of years, eukaryotic cells evolved multiple molecular mechanisms to maintain the integrity of their genome. Cell cycle checkpoints ensure the structural intactness of the DNA molecule, before a cell enters crucial cell cycle stages (e.g. S phase or mitosis). Once altered DNA activates a checkpoint, the cell cycle arrests. This allows the cell, depending on the extent of the damage, to repair the defect or to undergo apoptosis. If these processes are not properly executed transformation of a cell might lead to cancer in an organism.

2.1.1 The cell cycle

The cell cycle contains the four phases: G1 (gap 1), S (synthesis), G2 (gap 2) and M (mitosis) (Figure 1). For a physiological cell cycle, the cell is reliant on a temporally well coordinated interplay between cyclin dependent kinases (CDKs) and cyclins. Forming a heterodimer, CDKs exhibit the catalytic activity, whereas cyclins represent the regulatory subunit. According to their name, cyclins are exclusively synthesized in the cell at the time of their requirement and are degraded as soon as the mission is accomplished, therefore regulating the kinase activity in a timely manner. Overall three interphase CDKs (CDK2, CDK 4 and CDK6), one mitotic CDK, the CDK1 and ten cyclins, arranged in the four classes A, B, D, and E, are important for cell cycle progression. To restrict the kinase activity, additionally to the transient cyclins, the INK4 family and the Cip/Kip proteins (e.g. the p21 protein) are two known families of CDK inhibitors, present throughout the cell cycle (reviewed in [1, 2]).

In response to a mitogenic stimulation, D-type cyclins are expressed. During G1, these cyclins associate with CDK4 and CDK6. The resulting activity promotes indirectly the expression of E-type cyclins, which then form a dimer with CDK2 in late G1. The CDK2-cyclin E complexes are only present for a short period during the cell cycle and are responsible for the transition from G1 to the S phase. To promote the S phase and also

partially G2, CDK2 further binds to A-type cyclins. On the G2/M boundary, the mitotic CDK1 associates with cyclin A to onset mitosis. During mitosis itself, upon degradation of cyclin A, cyclin B overtakes the activation of CDK1 and can therefore drive the cell through mitosis until the end of metaphase (reviewed in [1, 2]).

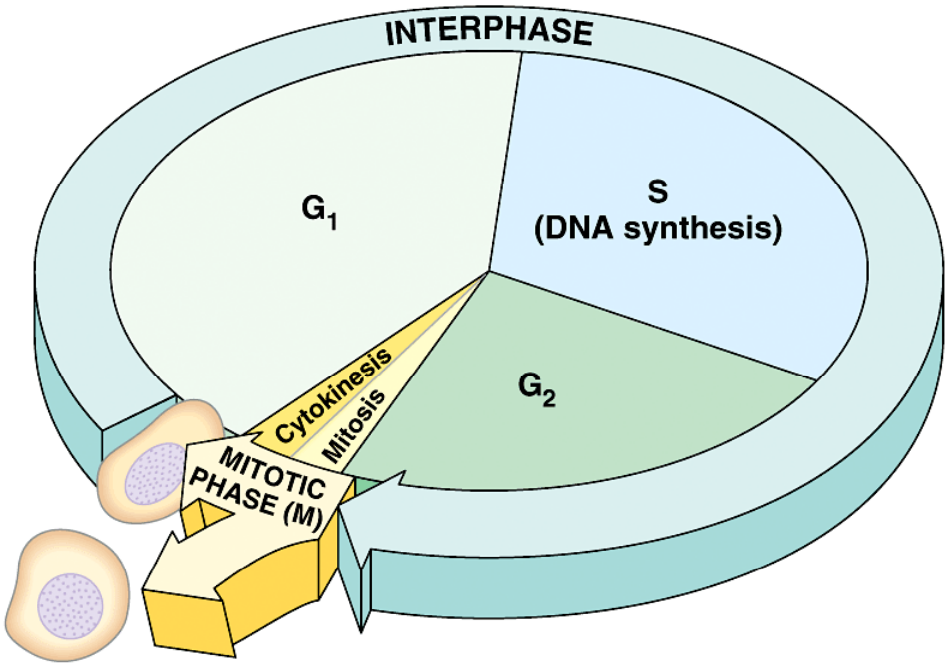


Figure 1: The cell cycle phases. (Copyright © Pearson Education, Inc.). For details see text.

2.1.2 Interphase checkpoints

The biochemical cascade that leads to DNA damage induced cell cycle delay or arrest can be broadly divided into five categories. First, *sensor* proteins monitor the genome for any alterations and initiate the DNA damage response (DDR). In mammalian cells, the Rad9-Hus1-Rad1 sliding clamp complex, the Rad17-RFC clamp loading complex and the MRN nuclease complex appear to be sensors [3-5]. Second, checkpoint *mediators*, including MDC1, BRCA1, 53BP1 and Claspin fine-tune the DDR [5, 6]. Third, ATM and ATR, so called *signal transducing kinases* of the PIKK (phosphatidylinositol 3-kinase-like kinases) family further amplify and propagate the signal downstream [3, 7]. ATM responds to DNA double-strand breaks (DSB), while ATR is activated upon single-strand DNA. Fourth, the *effector kinases* Chk1 and Chk2 connect the checkpoint with the cell

cycle machinery, whereas, Chk1 and Chk2 are specific substrates of ATR and ATM, respectively [6]. Fifth, as final targets of the checkpoint cascade, a broad spectrum of cellular proteins serve as *effector proteins*.

2.1.2.1 G1/S checkpoint

The G1/S checkpoint is responsible to ensure that only cells with undamaged DNA enter the S phase. If DSB occur during G1, the checkpoint activation is mainly due to the ATM-Chk2 axis. This is due to the fact, that during G1, no sister chromatid is available for homologous recombination (HR), so nonhomologous end joining (NHEJ) is the repair mechanism of choice. But in contrast to HR, NHEJ does not generate single-strand DNA by DSB end processing, therefore ATR-Chk1 is much less activated.

The G1/S checkpoint targets two distinct effector proteins working in parallel branches. The fast branch includes phosphorylation of the phosphatase Ccd25A on multiple serine residues by Chk1/Chk2. This leads to subsequent polyubiquitination and proteasome-mediated degradation of Cdc25A [8, 9]. Thereby, activating dephosphorylation of CDK2, the catalytic subunit of the CDK2-cyclin E and CDK2-cyclin A complexes, is prevented [10, 11]. As a consequence of this inhibition, formation of the replisome is avoided and DNA synthesis can not start [12]. This pathway, targeting Cdc25A, reacts rapidly upon DNA damage and is therefore important to initiate the cell cycle delay. However, as a transient pathway, it is only effective for a few hours [10, 11, 13].

Whereas this first branch works via post-translational modification, the second branch, responsible for prolonged maintenance of the cell cycle arrest, requires transcription dependent on the tumor suppressor protein p53 [14, 15]. Upon checkpoint activation, p53 is directly phosphorylated and thus stabilized, by both Chk1/Chk2 and ATM/ATR [7]. Additionally, ATM/ATR inhibit the ubiquitin ligase Mdm2 that guarantees under physiological conditions a rapid p53 turnover [16, 17]. The subsequent accumulation of p53 leads to the transcription of the key effector p21, a cyclin dependent kinase inhibitor, which inhibits the CDK2-cyclin E complex, therefore blocking the G1 to S transition [18].

2.1.2.2 Intra-S phase checkpoint

In contrast to the G1/S checkpoint, the fast intra-S phase checkpoint is only capable to induce a transient and reversible cell cycle delay, since it is p53 independent [3, 7, 14]. Collapsed or stalled replication forks often expose single-strand DNA and hence mainly activate the ATR-Chk1 axis [14]. The subsequent phosphorylation and proteolysis of the Cdc25A phosphatase leads to an inhibition of the CDK2-cyclin E/A complex, which further prevents the initiation of new origins of replication [10-12, 19].

However, the ATM-mediated phosphorylation of NBS1, a member of the MRN complex, and the cohesin protein SMC1, represents a parallel branch to the Cdc25A-degradation pathway [7, 20-23]. Whereas NBS1 is recruited early to sites of DNA damage in an ATM-independent manner to process the DSB ends [24-26], subsequent phosphorylation by ATM introduces NBS1 in the intra-S phase checkpoint pathway [27]. The following downstream events, mediated by NBS1/SMC1, leading to inhibition of the DNA replication, remain elusive.

2.1.2.3 G2/M checkpoint

To ensure that no altered DNA is passed onto daughter cells during mitosis, the G2/M checkpoint arrests all cells harboring a damaged genome in late G2 phase [28, 29]. Like the G1/S checkpoint, the cell cycle arrest in G2 is on one hand due to post-translational modifications and on the other hand upon activation of transcription programs. DSBs arising in G2 are mainly repaired by HR upon activation of ATM and ATR.

The major downstream target of ATM-Chk2/ATR-Chk1 is the mitosis-promoting activity of the CDK1-cyclin B complex. Interestingly, in this context, not only Cdc25A is of importance, but all three members of the Cdc25 phosphatase family, being Cdc25A, Cdc25B and Cdc25C. They were shown to be positive regulators of the CDK1-cyclin B kinase [30, 31]. Cdc25A becomes most likely degraded as described in the fast G1/S and intra-S phase checkpoint [30-32]. The mechanism for Cdc25B is less clear. Upon UV light induced damage, the mitogen-activated kinase p38 becomes active, phosphorylates Cdc25B [33] and promotes binding to the 14-3-3 protein, thus preventing access of

substrates to Cdc25B [34]. Following to phosphorylation by ATM-Chk2/ATR-Chk1, Cdc25C is inhibited and/or undergoes subcellular sequestration [3, 29]. Additionally but independent form the signal transducing kinases and the effector kinases, the checkpoint mediators 53BP1 and BRCA1 also contribute the G2/M checkpoint activation [35-38].

To prolong and to maintain cell cycle arrest, the p53 pathway is activated. Beside the expression of p21, the up-regulation of additional transcriptional targets of p53, such as the 14-3-3 protein and GADD45 alpha (growth arrest and DNA-damage-inducible 45 alpha) is required [29, 39]. However, tumor cells defective for p53 are still able to induce and maintain a cell cycle arrest in G2 after DNA damage. This suggests that alternative pathways, such as the BRCA1-mediated expression of p21 and GADD45 alpha, cooperate with the p53-cascade [29].

2.1.3 Spindle assembly checkpoint (SAC)

In contrast to the checkpoints mentioned above, the mitotic checkpoint does not monitor the integrity of the DNA itself, but ensures a proper attachment of the spindle microtubules to the kinetochores. SAC likely monitors both tension at the kinetochores and occupancy of the microtubules. Hence, a correct bipolar orientation of the chromosomes on the metaphase plate results, representing an important step to avoid aneuploidy. A single unattached kinetochore is sufficient to arrest a cell in mitosis. When the checkpoint is activated, SAC prevents the anaphase onset by inhibiting the key target Cdc20. When Cdc20 is not inhibited, it activates the anaphase promoting complex/cyclosome (APC/C). APC/C subsequently further acts as an E3 ubiquitin ligase that targets at least the two major inhibitors of mitotic exit securin and cyclin B for proteasomal degradation thus allowing the anaphase to start (reviewed in [40-42]).

2.2 DNA double-strand breaks

DSB are considered to be the most harmful DNA lesions, since a single DSB is sufficient to induce apoptosis [43]. DSBs occur from endogenous as well as exogenous attacks on the DNA molecule. Proper repair is essential for the genome stability, because alteration

or loss of DNA fragments can provoke apoptosis but also carcinogenesis by activating oncogenes on one side and inactivating tumor suppressor genes on the other. However, DSBs do not only arise accidentally. Eukaryotic cells also produce DSBs on purpose by inducing genes, encoding for specific endonucleases. DSBs are also physiologically important for genetic mixing and proper chromosome segregation during meiosis [44] and for producing a diverse immune repertoire in the context of V(D)J and class-switch recombination [45].

For accurate repair of DSBs, the cell can choose between two distinct repair pathways: NHEJ and HR.

2.2.1 DNA double-strand break repair

2.2.1.1 Nonhomologous end joining

NHEJ fulfills its repair function by re-ligating the newly emerged DNA ends together. Even though NHEJ is considered to be more error-prone than HR [46, 47], it is a very powerful DSB- repair pathway, since it has the ability to ligate any kind of DSBs, independently of the presence of homologous sequences and can therefore act in all stages of the cell cycle.

Beside the structural stabilization of the free ends, a minimum of base pairing between the two DSB ends is essential for proper ligation. To optimize base pairing, terminal base degradation is necessary [48, 49]. Prior to ligation, the remaining gaps are filled by DNA synthesis. To perform this complex reaction, many protein factors are needed on the DNA damage. In common for all NHEJ events is the presence of the core NHEJ machinery, which is composed of three complexes: The MRN, the Ku and the DNA ligase 4/Lif1 complex [50].

The yeast MRX complex, composed of Mre11, Rad50, and Xrs2, is essentially involved in NHEJ. The analogous complex in vertebrates, the MRN complex (MRE11, RAD50, NBS1) is strongly supposed to be involved in the NHEJ reaction but so far, no definitive evidence is given. The yeast MRX was shown to be one of the first complexes binding

DSB ends after their appearance [51]. Rad50 harbors a high-affinity DNA binding domain and is therefore able to bridge the DNA lesion, thus ensuring the proximity between the DSB ends [52-54]. This facilitates the coming base pairing and ligation. The *in vitro* shown nucleolytic activity of Mre11 could participate in DSB end processing in order to optimize base paring but it is not essential [55, 56]. Finally, Xrs2 acts as a regulatory subunit of the yeast MRX complex. It is phosphorylated upon DSB formation and therefore part of the damage-signaling pathway [57]. Together with Rad50, Xrs2 also influences MRX substrate binding [58]. Additionally, Xrs2 interacts with Lif1, a cofactor of DNA ligase 4. Consistent with this observation, *in vitro* experiments showed that MRX stimulates the ligation by the use of the DNA ligase 4 complex [59]. Overall, the essential function of the MRX complex during NHEJ seems to be tethering the DSB ends together thus recruiting the ligase complex.

The heterodimeric Ku complex, composed of yeast (y) Ku70/human (h) KU70 and yKu80/hKU80, is required for NHEJ [48]. Whereas in vertebrates Ku is part of the DNA-dependent protein kinase (DNA-PK), the catalytic subunit of this large complex, the DNA-PK_{cs}, is as well obligatory for NHEJ [60]. The entire complex participates in NHEJ as a DNA end-bridging factor [61]. Since this function is analogue to the one of the yeast MRX complex, it might be an explanation why the MRN complex in higher eukaryotes in not obligatory required during NHEJ. Ku itself is known to bind double-strand DNA and to be in direct contact with DNA ligase 4. Therefore, it may play a role as stabilizer and/or stimulator for the subsequent ligation reaction [62, 63]. Yeast *S. cerevisiae* lacking of yKu70 is known to have a faster DSB end degradation, suggesting that the Ku complex has a protective function at the double-strand end [64, 65]. In summary, the Ku complex has, as well as the MR(X)N complexes, a structural function in order to stabilize DSB ends and additionally thus preventing its degradation. Furthermore, the interaction with DNA ligase 4 may be to stabilize the protein and/or to stimulate its enzymatic activity.

Regarding ligation, the NHEJ ligase complex, consisting of DNA ligase 4/DNA ligase IV [47, 66], Nej1/XLF [67, 68] and the obligatory cofactors Lif1/XRCC4 [69, 70], is

essential. Lif1/XRCC4 and Nej1/XLF are so far known to have a stimulating function on the ligase activity of DNA ligase 4 and DNA ligase IV, respectively [66, 71]. As part of this complex, DNA ligase IV shows a high degree of substrate flexibility. Consequently it is able to perform ligation across small gaps as well as one strand independent of the other one [72]. In combination with the Ku complex, even mismatched or incompatible DNA ends can be ligated [72, 73]. All of this argues for the capability of the NHEJ reaction to repair many kind of DSB.

Depending on the type of damage, the DSB ends have to be processed prior to ligation. If terminal nucleotides are damaged or modified and do not show anymore 5' phosphates and 3' hydroxyls, the ligation reaction is impaired. For processing, proteins like the mammalian polynucleotide kinase (PNK) [74, 75], which harbors a 5' kinase and a 3' phosphatase activity or Aprataxin [76, 77], which is able to remove adenylate groups from 5' phosphates, are recruited to the damage site, mostly via interaction with XRCC4. Damaged bases or mismatches are corrected by DNA nucleases. In human cells, the nuclease Artemis is known to act as a 3' or 5' flap endonuclease at DSB ends [78, 79]. Finally, remaining single-strand gaps are filled by DNA polymerases of the X family, most likely DNA polymerases λ and μ and the terminal deoxynucleotidyl transferase (TdT) [80-82]. The high degree of substrate flexibility of these enzymes emphasizes the potential of NHEJ to repair DSBs with various structures at their end.

2.2.1.2 Homologous recombination

HR represents the most faithful DSB repair mechanism. For successful repair, HR is reliant on the availability of homologous sequences between the DNA double-strand harboring the damage and another intact DNA molecule, in the majority of cases the sister chromatid and seldom the homologous chromosome [83] (Figure 2).

All HR reactions have the first and essential step in common, namely the 5' resection of DSB ends [84, 85]. The resulting 3' single-strand overhang is now capable of searching for sequence homology, by invading the duplex containing the homology and facilitating DNA synthesis [83]. The nucleolytic enzymes performing this 5' degradation are still not

completely known. Mre11, exhibiting a 3' → 5' exonuclease activity, is more supposed to 'clean' the DSB ends from modified or damage bases [55, 86], therefore, serving as a forerunner of the 5' DSB end resection. Also the endonuclease Sea2/CtIP, which is known to be involved in DSB end processing together with the MR(X)N complex [87, 88], is mostly related to processing hairpin structures and meiotic DSB *in vivo* but not to 5'-ended strand degradation [89]. However, a known active player in this process is the 5' → 3' exonuclease Exo1/EXO1 [86, 90]. Apparently it is not the only one, because cells lacking of both Mre11 and Exo1 nuclease activities are still able to perform 5'-ended strand resection [91].

The following formation of a displacement-loop (D-loop) represents a central step of all HR mechanisms. When after unsystematic collisions between DNA molecules, the homologous sequence is finally found, the invasive strand has to squeeze itself between the intact double-strand DNA, thereby displacing one strand and pair with the other. The resulting DNA heteroduplex is then called D-loop. In detail, shortly after the 5' end resection, the resulting 3' single-strand is bound by the heterotrimeric replication protein A (RPA) [51, 92]. RPA interacts with Rad52 in yeast [93] or BRCA2 in vertebrates [94, 95]. Subsequent loading of the recombinase protein Rad51 on the single-strand DNA, forming a Rad51 filament, allows then strand invasion and exchange during homologous recombination [96]. Depending on the further HR mechanism used, the DNA heteroduplex can resolve in end products containing reciprocal exchanges (crossovers) or not.

2.3 DNA replication in eukaryotes

To guarantee that every daughter cell receives a complete and correct set of chromosomes, a proper duplication of the genome is a prerequisite. Due to the fact that errors, if not repaired, are passed on to the next generation, this complex mechanism is tightly controlled. 10 hours per cell cycle (40%) are spent exclusively for the S phase for replication. Here the focus is put on the initiation of DNA replication and hence the

recruitment of important factors, as this is the site of regulation and relevant for the presented work.

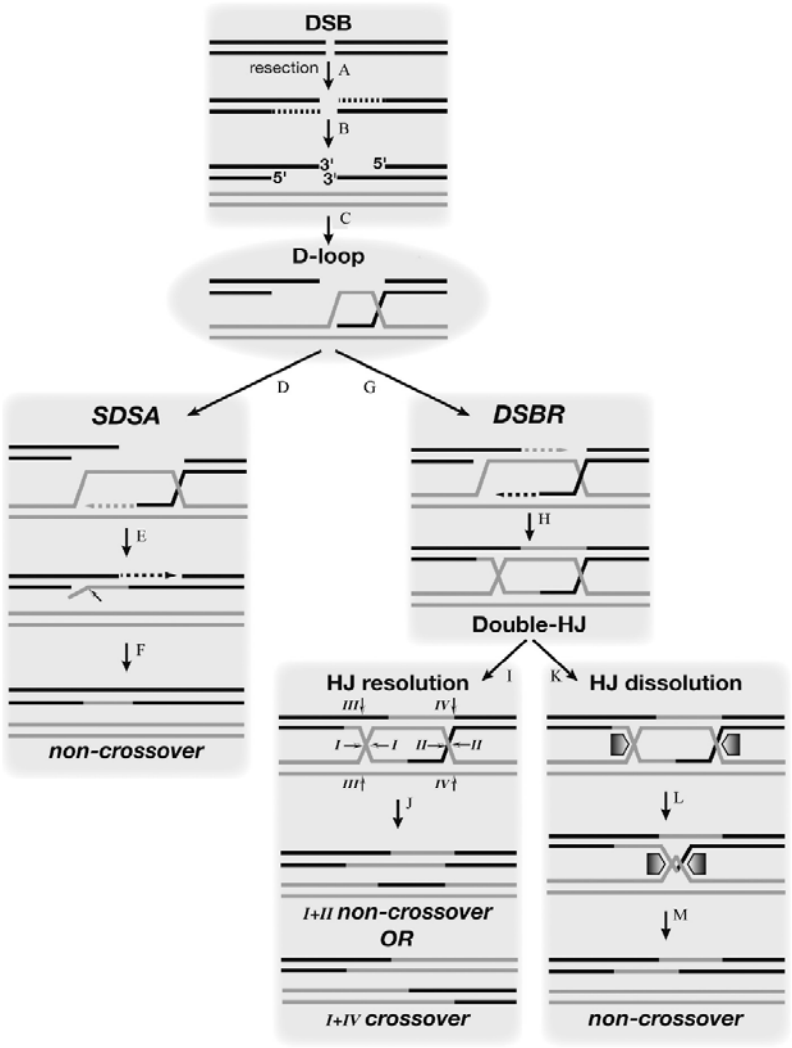


Figure 2: Recombination-mediated DSB repair by synthesis-dependent strand-annealing (SDSA) and double-strand break repair (DSBR). (A) DSB 5'-end resection (dashed black lines). (B) Search for a homologous template (grey). (C) Strand invasion and D-loop formation. (D) SDSA pathway. (E) Strand displacement of the elongated invasive strand (dashed grey arrow) and annealing to the other DSB end. (F) Cleavage of non-homologous sequence (arrow with black and white arrowhead), gap filling (dashed black arrow) and ligation. (G) DSBR pathway. (H) Elongation (dashed black and grey arrows) and ligation of invasive strands: double-Holliday junction (HJ) formation. (I) HJ resolution. (J) Differential cleavages I, II, III and IV are indicated (arrows with black and white arrowheads). Cleavages I+II (or III+IV, not represented) produce a non-crossover. Cleavages I+IV (or II+III, not represented) produce a crossover. (K) HJ dissolution by human BLM helicase and TOPO III alpha topoisomerase. (L) HJ branch migration and (M) strand decatenation (pentagonal shapes) produce a non-crossover. Reproduced from [83].

2.3.1 Prior to S phase

After a cell has decided to divide, members of the AAA ATPase family (ATPases associated with various cellular activities) were found to play a key role in the initiation of DNA replication [97]. The first step is the formation of the pre-replicative complex (pre-RC) at the origin of replication (Figure 3), which can only occur during G1 phase [98-103]. This starts with the binding of the origin recognition complex (ORC) onto the DNA. Three out of the six subunits of the ORC are considered to be AAA ATPases. Serving as a putative platform, ORC facilitates the assembly of Cdc6 (AAA ATPase), Cdt1 and the Mcm2-7 complex to the origin, which altogether compose the pre-replicative complex. The initiation factors Cdc6 and Cdt1 help to load the Mcm2-7 heterohexamer consisting of six AAA ATPases. They likely form the replicative DNA helicase. Due to the fact that replication is bi-directional, the Mcm2-7 complex is most probably loaded at least in duplicate at the origin of replication [104]. The origin is now licensed for replication [105, 106].

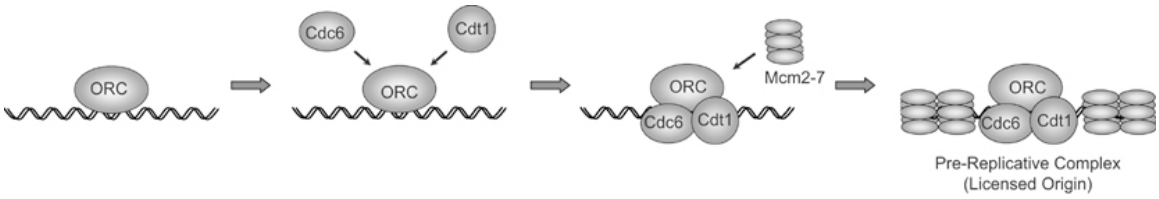


Figure 3: Assembly of the pre-replicative complex. Stepwise recruitment of pre-RC components to the origin of replication during G1. Multiple Mcm2-7 helicase complexes are loaded. Reproduced from [107].

Even though every pre-RC is intrinsically able to initiate DNA replication, only a small amount is allowed to fire origins [108-111]. The additional pre-RCs represent a backup for dormant origins, which only fire if neighboring origins fail to initiate replication [112, 113]. In this way the cell ensures, that an adequate number of origins are activated even under various physiological conditions.

Whether an origin is fired as an early or as a late origin, is decided right after pre-RC formation in early G1 phase at the so called timing decision point (TDP) [110, 114, 115]. The reason for this temporal distribution is an area of intensive current research.

2.3.2 Intra S phase

To fire the early origins of replication, the pre-RCs have to be modified into bi-directional replication forks while entering the S phase. To achieve this transformation, a fascinating interplay between several replication factors proceeds. For coordination of these processes, CDKs and the Dbf dependent kinase (DDK) are required [116-118]. In opposite to the assembly of pre-RCs, which occurs simultaneously and independent from the temporal characteristics of the origins, the conversion into replication forks only takes place directly before activation [117-119].

To avoid genome instability and chromosome breakages, it is essential that during initiation of replication each origin fires only once per cell cycle. Participating in this task, CDKs harbor two crucial functions to prevent re-replication. On one hand, the CDK is directly involved in the activation of origins, which simultaneously leads to the disassembly of the pre-RC and on the other hand, high CDK activity inhibits the formation of new pre-RCs in S phase [98, 99]. In higher eukaryotes, the protein Geminin represents a second mechanism to avoid re-licensing and re-replication. Appearing as a dimer, Geminin physically interacts with Cdt1 [100-102, 120]. Even though loaded to chromatin, Cdt1 in complex with Geminin is not able anymore to recruit Mcms, likely as a result of steric hindrance [121-123].

Once the Mcm helicases start to work in both directions, single-strand DNA is stabilized by RPA and replicative DNA polymerases α, δ and ε together with the auxiliary factors proliferating cell nuclear antigen and replication factor C are loaded and the replication process itself can start (for more details see [124]).

2.4 Ubiquitin and small ubiquitin-like modifiers

2.4.1 Ubiquitination

Ubiquitin is a small polypeptide of 8.5 kDa, consisting of 76 amino acid residues. The post-translational modifications of proteins by ubiquitin conjugation have reached a broad significance in the last years. Since the covalent binding to the substrate is reversible, such as for phosphorylation, ubiquitination represents an ideally regulatory pathway. To bind ubiquitin to the substrate, an ATP dependent cascade of three distinct enzymes has to be run (Figure 4, upper part). First, an E1 activating enzyme activates ubiquitin, by ligating it covalently to its active cysteine residue. The active ubiquitin is then further transferred to an E2 conjugating enzyme. Finally, ubiquitin is ligated to an acceptor lysine of the target protein, in a process stimulated by an E3 ligase. According to the high number of different E3 ligases expressed, harboring substrate-binding sites, the substrate specificity of the ubiquitin modification is provided by the E3 ligases. Resulting from ubiquitination, a target protein can be mono- or polyubiquitinated. The best studied modification so far, is the lysine 48 (Lys48) polyubiquitin chain, meaning that every added ubiquitin molecule binds with its C-terminus to the lysine residue on position 48 of the previous ubiquitin molecule. Proteins marked by Lys48-polyubiquitination are designed for proteasomal degradation by the 26S proteasome. Monoubiquitination and polyubiquitination via lysine 63 (Lys63) is important in many other regulatory cellular processes, such as e.g. DNA repair, gene expression and endocytosis. Once the signal performed by ubiquitin conjugation is not of use anymore, ubiquitin-specific proteases (UBPs) reverse the modification (reviewed in [125, 126]).

2.4.2 SUMOylation

Very similar to the ubiquitin pathway, reversible conjugation of proteins with the small ubiquitin-like modifier (SUMO) emerged as a novel regulatory pathway for protein function and localization. Also SUMO is conjugated to its substrate via the ATP dependent cascade, consisting of E1, E2 and E3 enzymes (Figure 4, lower part). In contrast to the complexity of the ubiquitin system, the SUMO pathway is simpler, involving only one E1, a single E2 and a few E3 enzymes, even SUMOylation also

targets a large number of substrates. Conjugation of SUMO molecules to proteins mostly occurs on lysine residues within a consensus sequence. Opposite to yeast that only knows one SUMO-type, vertebrates have three SUMO variants, whereas only SUMO-2/3 can form polySUMO chains, but not SUMO-1. Compared to polyubiquitin chains, only little is known about the function of chains built by polySUMOylation. Given that SUMOylation is reversible, ubiquitin-like protein-specific proteases (ULPs) are able to hydrolyze the conjugates (reviewed in [125, 127]).

Interestingly, ubiquitin and SUMO do not only act in a similar way, they also interact functionally. Thus, polySUMOylation of a certain protein recruits an E3 ubiquitin ligase for polyubiquitination and further proteasomal degradation. Additionally, ubiquitin may modify SUMO molecules, possibly resulting in mixed ubiquitin-SUMO chains with so far unclear relevance [127]. Very recently SUMO was found to be the crucial regulator of ubiquitination in DNA repair [128, 129].

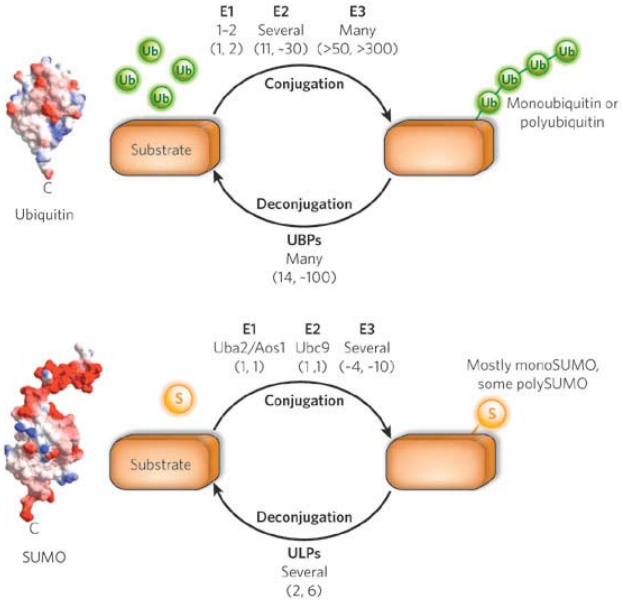


Figure 4: Schematic diagram of ubiquitin and SUMO modifications. Ubiquitin (Ub, top pathway) and SUMO (S, bottom pathway), known as modifiers, are small polypeptides that are usually conjugated to internal lysine residues of target proteins through their C-terminal glycine residues, forming isopeptide bonds. Both modifications are reversible. Modification can be by a single modifier or by a chain of covalently linked modifiers (polyubiquitination or polySUMOylation). The number of enzymes in the yeast *Saccharomyces cerevisiae* and in humans, respectively, are shown in parentheses. UBPs, ubiquitin-specific proteases; ULPs, ubiquitin-like protein (SUMO)-specific proteases. Reproduced from [125].

2.5 AAA ATPase p97/Valosin-containing protein (VCP)

The chaperone protein p97/VCP is an essential, ubiquitous and highly abundant protein, representing more than 1% of all cellular proteins. As an AAA ATPase (ATPases associated with various cellular activities) it contains two characteristic AAA modules, D1 and D2. Each consisting of a Walker A (ATP binding), Walker B (ATP hydrolysis) and a second region of homology (SRH) motif (reviewed in [130]).

2.5.1 History of p97/VCP

Before p97/VCP was described in mammals in the late 1980s, the yeast homologue Cdc48p was found a few years earlier in 1982 [131]. While screening mutagenized yeast colonies, the cell-division-cycle gene Cdc48 mutant stuck in mitosis with an undivided nucleus and microtubules spreading unsystematic throughout the cytoplasm [131]. Later valosin itself, a 25-amino-acid peptide, was isolated from porcine intestinal extracts [132]. 1987, it was shown that valosin was just a degradation product deriving from a hitherto unknown protein. Thereupon, the valosin-containing protein, VCP was predicted for the first time in mammals [133]. Initially, it was assumed to be a precursor protein but as non of the characteristic sequences were found, it was strongly believed to be an exclusive cytoplasmic protein [133]. Further immunoprecipitation of subcellular fractions revealed an immunoreactivitiy of >95% in the cytoplasm [133]. Today, two decades later, p97/VCP is also known as VAT in archaebacteria [134], Cdc48p in yeast [135], TER94 in *Drosophila* [136], p97 in *Xenopus* [137] and p97/VCP in plants and mammals [133] and therefore represents one of the very highly evolutionary conserved proteins.

2.5.2 Structure of p97/VCP

The 97 kDa protein is a member of the type II AAA ATPases and can be divided into four domains: N, D1, D2 and the C-terminal extension [138, 139]. To fulfill its biological function, p97/VCP assembles to a highly stable, nucleotide-independent, barrel-like, homo-hexameric protein, existing of two ring-shaped layers formed by the two AAA modules, D1 and D2 [139-141] (Figure 5). Furthermore, the hexamer exhibits a central “pore” with a complex interior shape [138]. The wider “ring” is made of the N-D1

domains, while the D2 domains occupy the smaller one. Cryo-EM analysis illustrated, that the p97/VCP hexamer undergoes remarkable conformational changes during the ATPase cycle (binding of ATP, ATP hydrolysis, P_i release and finally ADP release). Both, the D1 and even more the D2 ring become more compact upon ATP binding, while the D2 ring additionally starts to rotate forth and back during the ATPase cycle [142]. In summary, ATP hydrolysis, in combination with conformational changes, generates the needed mechanical force for the p97/VCP dynamics. Despite this knowledge, it is still under investigation, which model in the end is the right one for the dynamics of p97/VCP.

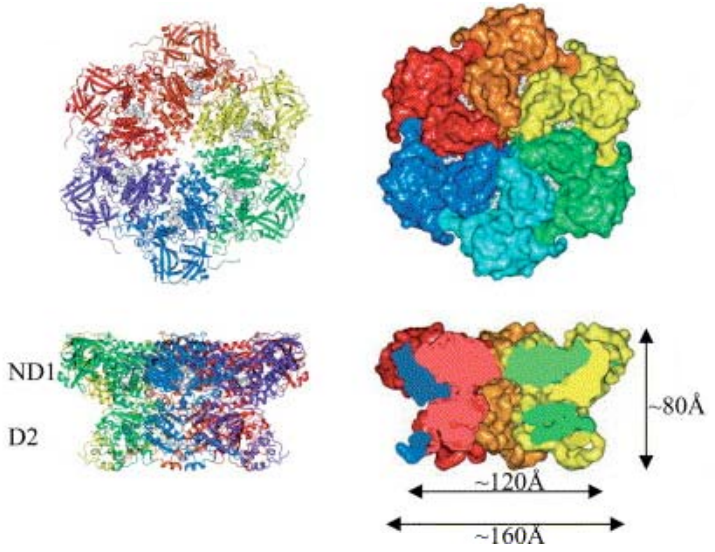


Figure 5: Overall structure of the p97. (A) Ribbon representations (top and side views) of the p97 hexamer with each protomer coloured differently, wider ring is the N–D1. (B) Molecular surface representations of the p97 hexamer showing the overall shape and dimensions of the hexamer body. Adapted from [143].

2.5.3 Functions of the p97/VCP domains

p97/VCP contains three functional domains called N, D1 and D2, as well as a C-terminal extension region (Figure 6).

2.5.3.1 N domain

Crystal structure analysis of N-D1 revealed that the N domain is located in the periphery of the D1 ring [139]. As the least conserved region, the N domain binds directly to cofactors/adaptors and thereby mediates the target specificity of p97/VCP [144]. Even though the N domain is neither needed for oligomerization nor for ATPase activity, while working together with D1 and D2 domains, it is involved in mediating nucleotide-induced conformational changes [142]. According to the importance of the N domain,

abnormalities are expected to result in pathological phenotypes. Indeed, autosomal-dominant single amino-acid substitutions, within the N domain and the D1 ring, are responsible for the clinical picture of the rare hereditary inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia (hIBMPFD) [145].

2.5.3.2 D1 domain

Shown by crystallography and biochemical studies, the D1 domain is the major domain responsible for hexamerization of p97/VCP [139, 146]. Many other AAA proteins also form oligomers if nucleotides are available, but dissociate into mono- or dimers in the absence of nucleotides. In contrast, p97/VCP forms a surprisingly stable hexamer, independent of the presence of nucleotides [146, 147]. As an ATPase, containing Walker A and Walker B motifs, which mediate ATP binding and hydrolysis, respectively, the D1 apparently has a minor importance as demonstrated by *in vitro* studies [148, 149].

2.5.3.3 D2 domain

Despite the high sequence similarity between the D1 and D2 domain, the D1 domain promotes homo-oligomerization, whereas the second AAA module D2 performs the major ATPase activity [148, 149]. Neglecting the physiological conditions, the D1 ATPase shows a heat-enhanced activity around 50°C, which is speculated to be due to a more relaxed D1 ring upon heat treatment. Therefore, the enzymatic site becomes more flexible and accessible to the solvent, where ATP can be found [148].

2.5.3.4 C-terminal extension

Less is known about the C-terminal extensions. Due to its highly flexible nature, it is more difficult to characterize it structurally and functionally [147]. Of functional importance is the major tyrosine (Tyr) phosphorylation site at the very C-terminus, Tyr805 [150]. For example, the transitional assembly of p97/VCP to the endoplasmic reticulum is regulated by tyrosine phosphorylation [151]. In yeast, Cdc48p is translocated into the nucleus upon cell cycle-dependent tyrosine phosphorylation of the C-terminus. The phosphorylation induces a conformational change, resulting in the exposure of the nuclear import signal sequence at the N domain [152]. Short, tyrosine phosphorylation of the C-terminal extension serves as a localization signal.

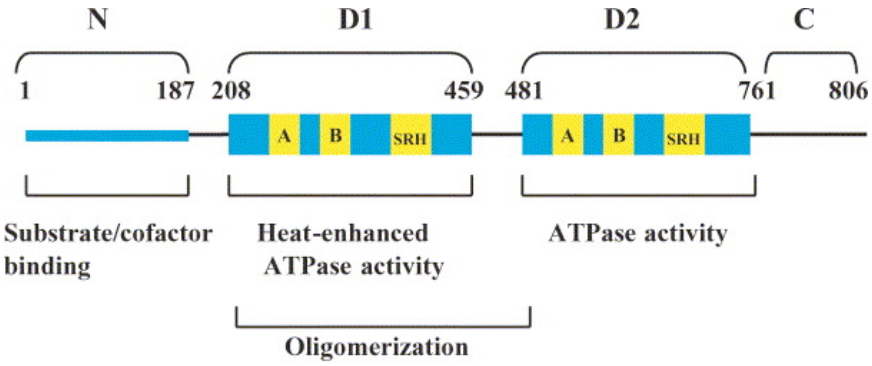


Figure 6: Functional domains of p97/VCP. Based on the reported *in vitro* and *in vivo* studies, the major function of each domain in VCP is summarized. A, B, and SRH indicate the Walker A, Walker B, and the second region of homology motifs, respectively. Adapted from [130].

2.5.4 Functions of the p97/VCP hexamer

p97/VCP is a highly abundant chaperone and involved in many cellular activities. Assembled as a hexamer, p97/VCP functions in an adaptor-dependent manner. Therefore, the specificity of action is given by different adaptors bound to the N domain. In complex with a distinct adaptor, p97/VCP acts as an ubiquitin-selective segregase, thus recognizing polyubiquitinated substrates. A very well understood interaction is the one of p97/VCP with its main adaptors, the binary complex Ufd1 and Npl4 [144]. Whereas both, Ufd1 and Npl4, bind to ubiquitin [149, 153-155]. Once bound to the substrate via its ubiquitin-related adaptors, p97/VCP can specifically extract the protein out of its environment, by hydrolyzing ATP. The further destiny of the substrate is often proteasomal degradation, but dependent of additional processing factors (e.g. de-ubiquitinating enzymes), different outcomes are possible. According to this model, p97/VCP emerged as a novel member of the ubiquitin system (reviewed in [130, 156]).

2.5.4.1 Endoplasmic reticulum-associated degradation (ERAD)

ER-associated degradation represents a rigorous protein quality control mechanism in the secretory pathway. Misfolded or improperly assembled membrane or luminal proteins are extracted from the ER, retro-translocated into the cytoplasm and subsequently degraded by 26S proteasomes [157-162].

Early observations suggested that the energy needed to extract the proteins from the ER, was provided by ATP hydrolysis of the proteasome itself [163]. This assumption was later proven invalid, as soon as yeast studies showed that proteasomes are dispensable for retro-translocation. But even more striking was the fact, that the same studies revealed that the trimeric Cdc48p$^{Ufd1/Npl4}$ complex was a new player, involved in the export of polyubiquitinated improperly folded ER proteins [164-167]. Ongoing research led to the current model that Cdc48p, together with its two ubiquitin-related adaptor proteins Ufd1 and Npl4, binds to Lys48 polyubiquitinated substrates on the ER. During this step, a further substrate-processing cofactor called Ufd2 binds also to the Cdc48p$^{Ufd1/Npl4}$ complex, thus elongating ubiquitin conjugates on the respective protein substrate [168]. Once Cdc48p$^{Ufd1/Npl4}$ extracted the protein by the use of ATP hydrolysis, additional substrate-delivery factors such as Rad23 and Dsk2 come in to play. They are associated with Ufd2 and together they finally target the target protein to the proteasome for degradation [169, 170].

2.5.4.2 DNA repair

A first hint that p97/VCP plays a role in DNA repair was given when the physical interaction with the DNA repair protein BRCA1 was demonstrated both *in vivo* and *in vitro* [171]. A later study then evidenced the interaction between p97/VCP and a member of the RecQ Helicase family, the WRN protein [172, 173]. Additionally, phosphorylation of p97/VCP at the Ser784 upon DNA damage was described [174]. As responsible kinases, three important members of the phosphatidylinositol-3 kinase-related kinase (PIKK) family were shown; Namely ATR, ATM and DNA-PK, all kinases that manly target substrates involved in the DDR pathway.

2.5.4.3 Cell cycle progression

Originally Cdc48p was found in yeast as a protein important for cell division, even though the precise role remained unclear [131]. Further work then revealed involvement of p97/VCP in mitotic processes like spindle disassembly [175] and nuclear envelope reformation [176, 177]. Interestingly, a recent study in *C. elegans* indicated an implication of CDC48 together with its main adaptors Ufd1/Npl4 in DNA replication [178]. It is not clear yet how replication is affected, but depletion of CDC48$^{Ufd1/Npl4}$ led to

a decreased DNA synthesis, hypersensitivity towards hydroxyurea treatment and activation of the intra-S phase checkpoint. Due to the fact that some results were comparable to the loss of the replication initiation factors Cdt1 or Cdc6 [179], it was hypothesized that $CDC48^{Ufd1/Npl4}$ might be involved in initiation of DNA replication.

2.5.5 p97/VCP and disease

hIBMPFD, hereditary inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia, is a rare but severe autosomal dominant pathology, which has recently been shown to be caused by mutations in the p97/VCP gene [145]. Arg155, located within the N domain, is by far the most common amino-acid affected. How p97/VCP is altered by these mutations is not completely clear yet. Recent work showed that the spontaneous self-assembly of the hexamer is not impaired [180, 181]. A close analysis of the mutants $p97^{R155P}$ and $p97^{A232E}$ revealed a structural defect in the D2 ring, as it adopts a relaxed conformation compared to $p97^{WT}$ [181]. The same study showed also a 3-fold increased ATPase activity of the Arg155 mutant.

The pathogenesis of hIBMPFD is mainly attributed to the accumulation of proteinaceous inclusions in the cytoplasm and nuclei of degenerating myofibrils and dystrophic neuritis [182-185]. The ultrastructure and the composition of the inclusions, as well as the mechanism by which p97/VCP contributes to this accumulation, are poorly understood. It is hypothesized, that due to the ATPase hyperactivity p97/VCP gets too “hyperactive” and therefore looses the ability to properly bind to substrates. Since p97/VCP is an important mediator in the ubiquitin-proteasomal degradation pathway, this model could explain proteinaceous inclusion body formation.

Patients suffering from hIBMPFD can show three distinct clinical manifestations and hybrid forms of those (Figure 7). First, myopathy is the most abundant one, present in 80-90% of all affected people. Characterized by adult-onset (44 years) and muscle weakness often targeting the shoulder and hip girdle [186-188]. Second, in 43-51% of affected individuals, the Paget’s disease of bone is observed, whereby the spine, the pelvis and the skull are major targets of this feature, resulting in enlarged and deformed bones of fragile

quality [186-188]. Third, 31-37% of all hIBMPFD-patients suffer from frontotemporal dementia [186, 188]. Whereas memory remains relatively preserved, behavioral and language dysfunctions appear to be characteristic.

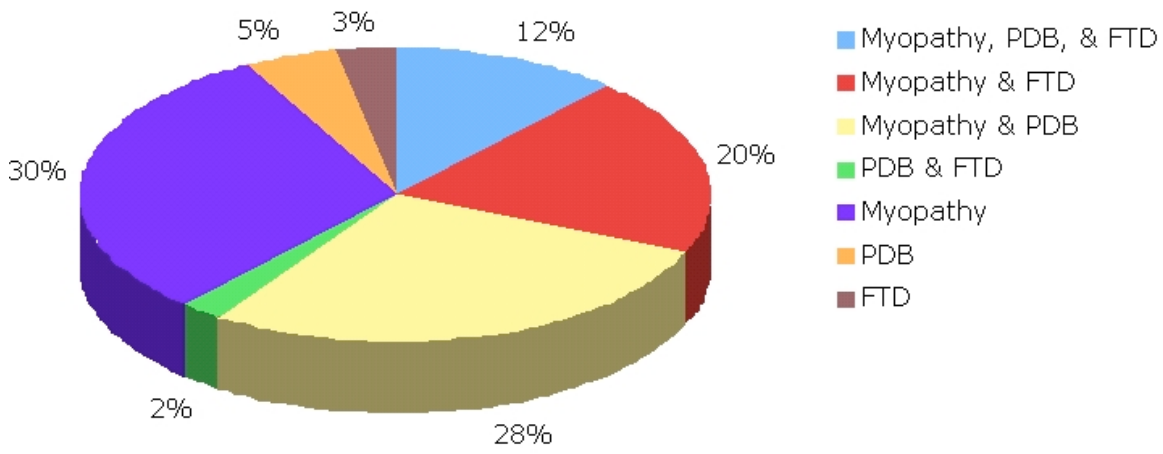


Figure 7: Penetrance of phenotypes associated with p97/VCP disease. PDB: Paget Disease of Bone, FTP: Frontotemporal Dementia. Reproduced form the Inclusion Body Myopathy Associated with Paget Disease of Bone and/or Frontotemporal Dementia GeneReview with permission from GeneTests (www.genetests.org) and University of Washington, Seattle.

3. Aim of the study

So far, there is evidence that p97/VCP is involved in the DNA damage response pathway, since previous studies showed interactions with the main protein altered in breast cancer, BRCA1 [171] or the WRN protein [172, 173]. Later experiments contributed additionally to this assumption, when they revealed that p97/VCP is phosphorylated upon DNA damage by the important DNA damage response kinases ATM, ATR and DNA-PK [174].

Consistent with these findings, knockdown results from our laboratory also strongly implicate p97/VCP in the DNA damage response pathway, more precisely, in DNA repair (Meerang, M and Ramadan, K, unpublished observations). Beside knockdown of p97/VCP, siRNA against Npl4, smc5 and mms21 was also part of the investigation. Smc5 together with smc6 form under physiological conditions a complex, which is known to be involved in important cellular activities requiring homologous recombination [189]. Mass spectrometry and immunoprecipitation results revealed smc5 as an interaction partner of p97/VCP (personal communication with Danilo Ritz and Hemmo Meyer, ETH Zürich). The E3 SUMO ligase mms21 is interesting, because it is also a component of smc5/6 complex [190] and therein facilitates homologous recombination [191]. Additionally, p97/VCP is supposed to be regulated by SUMOylation [192] and cells deficient for mms21, show a comparable phenotype to the one of p97/VCP knockdowns (unpublished observation from our laboratory). According to this knowledge, both smc5 and mms21 are potential substrates or regulators of p97/VCP.

To further verify the importance of p97/VCP in the DNA damage response, the cell cycle checkpoints became a topic of interest. According to preliminary side effect results from our laboratory, the G2/M checkpoint appeared to be a promising target. So the aims of the study were:

1. to characterize the influence of p97/VCP on the functional efficiency of the G2/M checkpoint and
2. to analyze whether known p97/VCP- adaptors or potential substrates are involved.

4. Materials and Methods

4.1 Buffers and solutions

Phosphate buffered saline (PBS):
137 mM NaCl
2.7 mM KCl
10 mM Na_2HPO_4
1.76 mM KH_2PO_4
pH 7.4 (adjusted with HCl)

PBS-T:
137 mM NaCl
2.7 mM KCl
10 mM Na_2HPO_4
1.76 mM KH_2PO_4
pH 7.4 (adjusted with HCl)
0.05% (v/v) Tween 20

Lysis buffer:
150 mM KCl
25 mM Tris-Base (pH 7.5)
5 mM $MgCl_2$
1 % (v/v) triton
2 mM β-mercaptoethanol
5 % (v/v) glycerol
1 µg/ml pepstatin
1 µg/ml leupeptin
1 µg/ml bestatin

Loading buffer (10x):
600 mM Tris-HCl (pH 6.8)
20 % (w/v) Na-dodecylsulfate (SDS)
20 % (v/v) glycerol
0.05 % (w/v) bromphenol blue
20 % (v/v) β-mercaptoethanol
25 mM dithiothreitol (DTT)

Solution B:
1.5 M Tris-HCl (pH 8.8)
0.4 % (w/v) SDS

Solution C:
0.5 M Tris-HCl (pH 6.8)
0.4 % (w/v) SDS
small amount bromphenol blue

Running buffer: 25 mM Tris-Base 192 mM glycine 0.1 % (w/v) Na-dodecylsulfate (SDS)	**Semi-dry transfer buffer:** 25 mM Tris-Base 40 mM glycine 10% (v/v) methanol
Primary antibody: Primary antibody diluted as outlined in chapter: 4.8 1 % (w/v) bovine serum albumin 0.05 % (v/v) NaN_3 in PBS	**Staining solution:** 2.5 % (v/v) propidium iodide (PI, 1mg/ml) or 1 % (v/v) 4′,6-diamidino-2-phenylindol (DAPI, 100x) in PBS
Secondary antibody: Secondary antibody diluted 1:10’000 1 % (w/v) bovine serum albumin in PBS-T	**FACS solution:** 1 % (v/v) PI (1mg/ml) 0.5 % (v/v) RNAse A in PBS
Blocking solution: 5% (w/v) milk powder in PBS-T	**Cell fixative:** 25% (v/v) acetic acid 80% 75% (v/v) methanol

4.2 Cell culture

Materials:

- Dulbecco's Modified Eagle Medium (DMEM) GlutaMAX-I (4.5 g/L glucose, no pyruvate), Gibco, Invitrogen (further referred to as “medium”)
- Tissue culture dishes, Ø 100 x 20 mm, TPP

Method:

Parental U2OS (osteosarcoma) cells were grown at 37°C in a 5% CO_2 incubator in medium supplemented with 10% fetal calf serum (FCS) and 100 U/ml penicillin-streptomycin.

4.3 Double thymidine block

Materials:

- Thymidine, cell culture tested, Sigma

Method:

To synchronize the cell cycle on the G1/S boundary, U2OS cells were grown until 30% confluency and subsequently incubated for 16 h in fresh medium containing 2mM thymidine dissolved in sterile distilled water. After this first block, the cells were washed twice with PBS and released in fresh medium for 8 h. The second block was carried out again with new medium containing 2mM thymidine. After another 16 h of blocking and the following two washing steps with PBS, the cells were released by adding fresh medium and therefore progressed synchronously through the cell cycle.

4.4 Small interfering RNA transfection

Materials:

- OPTI-MEM I, reduced-serum medium, Gibco, Invitrogen
- Lipofectamine™ RNAiMAX, Invitrogen
- Tissue culture dishes, Ø 60 x 15 mm, TPP
- siRNAi:
  - p97/VCP: Hs_VCP_6 HP Validated siRNA, SI03019681, Qiagen
  - Npl4: Hs_NPLOC4_1 HP siRNA, SI04211382, Qiagen
  - smc5: siRNAhsmc5, 5’ GCA GUG GAU UCA GGG UUG A dT dT 3’, Microsynth

- mms21: siRNAhmms21, 5’ CUC UGG UAU GGA CAC AGC U dT dT 3’, Microsynth
- non-silence: siLuciferase GL2 5’ CGU ACG CGG AAU ACU UCG A tt 3’, Microsynth

Method:

To achieve a successful knockdown of the protein of choice, incubation of the cell culture tissue with small interfering RNA (siRNA) has always taken place at least 48 h before the cells were harvested for analysis. Within these two days, the cells grew from a beginning confluency of 30% up to almost 100%. In combination with a double thymidine block, the transfection was performed at the very beginning of the first block.

First two tubes were prepared, one with 150 µl OPTI-MEM containing 160 nM siRNA and one with 147.6 µl OPTI-MEM + 2.4 µl of the transfection reagent RNAiMAX. For complex formation, these two tubes were poured together and incubated for 20 min at room temperature. To achieve a final siRNA concentration of 12 nM in the tissue culture dish itself, these 300 µl siRNA-mix were put in 1.7 ml medium. After at least 8 h, the transfection was considered to be complete and the medium was exchanged if indicated.

4.5 Mitotic index assay using metaphase chromosome spread

Materials:

- Nocodazole, Sigma
- RX-650 Cabinet X-Radiator System, Faxitron X-Ray
- Trypsin, 0.5 % (10x), 5 g/l, Gibco, Invitrogen
- Tissue culture dishes, Ø 60 x 15 mm, TPP
- Rotanta 460 centrifuge, Hettich
- MENZEL microscope slides, cut edges, double frosted ends, 1 x 76 x 26 mm, Menzel-Gläser
- Propidium iodide solution, 1.0 mg/ml in water, Sigma

- DAPI, Sigma
- Mowiol:

  24 g glycerol together with 9.6 g **Mowiol® 4-88 (Sigma)** was put in 24 ml ddH_2O. Additionally, 48 ml 0.2 M Tris-Base (pH 8.5) were added. Altogether was incubated on a hot plate (50 – 60°C) under permanent stirring until all mowiol powder was dissolved. Afterwards, the solution was centrifuged at 5'000 g for 15 min. The resulting supernatant was aliquoted and stored at – 20°C. Before use, mowiol was thawed at room temperature.

Method:

A mitotic index assay was established to get a quantitative picture of the G2/M checkpoint activity. To achieve this goal, almost confluent culture dishes of asynchronized or synchronized (6 h after release) cells were γ-irradiated with 3 Gy or 10 Gy. After irradiation, one hour was given for initial recovery before new medium containing 200 nM nocodazole (dissolved in DMSO) replaced the old one. Nocodazole is a drug that prevents the polymerization of the microtubules and therefore arrests cells in mitosis. This was important, since later the number of all cells that entered mitosis under various conditions was determined. The addition of nocodazole was considered to be the time point "0 h". Final samples for analysis were then harvested at the time points "6 h" and "9 h", respectively.

The "metaphase chromosome spread" was utilized to distinguish cells in mitosis from cells in the interphase by the use fluorescence microscopy, and therefore allowed to determine the mitotic index. U2OS cells were harvested at the distinct time points as follows. After the medium was collected, the culture dishes were washed with 4 ml PBS. This wash was collected as well. Afterwards, the cells were incubated with 2.5 ml trypsin (1x, 0.5g/L) for 5 min at 37°C to disassociate the cell monolayer from the cell culture dish. The trypsinized cells were then harvested and the whole cell suspension, consisting of the supernatant medium, the PBS-wash and the trypsinized cells, was spun down for 5 min at 1'000 rpm. All but approximately 300 µl of the supernatant was removed and the pellet was resuspended by gently tapping the tube with fingers. To get a hypotonic environment, 2 ml of 75 mM KCl, pre-warmed at 37°C, was slowly added before the samples were incubated for 10 min at 37°C. After incubation, previous to the next centrifugation step (5 min, 1'000 rpm), a few drops of the fixative, pre-cooled at 4°C,

were dripped into the tubes. Again, all but 300 µl of the supernatant was removed, the cells were gently resuspended and then the first fixation step was performed, by adding slowly 5 ml of the cell fixative pre-cooled at 4°C. After careful but proper mixing, the suspension was spun down (5 min, 1'000 rpm) and the fixation step was carried out twice more. The fixed cells can be stably stored at -20°C for several weeks.

To concentrate the cells, another centrifugation step (5 min, 1'000 rpm) was carried out, and all but 500 µl of the supernatant was removed. To get a nice spread on the glass slide, 20 µl (= 2 drops) of the concentrated suspension were dropped from a height of approximately 30 cm onto the glass slide, getting two separated, nicely spread drops. After the loft drying, the DNA was stained by pouring 300 µl staining solution (PI or DAPI) on the glass slide, covering the region where the two drops were placed. Prepared like this, the glass slides were stored at a dark place for 30 min. To finish the staining, the remaining staining solution on the glass slide was decanted, subsequently, the whole glass slide was shortly dipped in PBS, followed by a dip in distilled water. From now on, exposure to light was avoided. To protect the samples spread on the glass slide, 9 µl of Mowiol were placed on the more or less dry glass slide on each spot where the cells were dropped. As a final step, on each drop of Mowiol one cover slip was mounted. To achieve a proper adhesion, the glass slides were left for another 20 min at room temperature in the dark, before storing them at 4°C. After at least 1 h at 4°C, the samples were ready for fluorescence microscopy.

4.6 Fluorescence microscopy

Materials:

- BX51 fluorescence microscope, Olympus
- DP71 digital camera, Olympus
- cell^B software, Olympus

Method:

To determine the mitotic index, 300 cells per sample were enumerated. Nuclei harboring obviously condensed chromosomes were classified as cells in mitosis, whereas diffuse and homogenously stained nuclei were considered to belong to interphase cells.

4.7 Fluorescence Activated Cell Sorting (FACS) analysis

Materials:

- Flow Cytometer CyAn ADP 9, Beckman Coulter
- Rotanta 460 centrifuge, Hettich
- Tissue culture dishes, Ø 60 x 15 mm, TPP
- RNAse A, Roche Diagnostics

Method:

To determine the cell cycle stage, the cellular DNA content was measured by using FACS analysis. Therefore, U2OS cells were harvested as follows: after the medium was collected, the culture dishes were washed with 4 ml PBS. This wash was collected as well. Afterwards, the cells were incubated with 2.5 ml trypsin (1x, 0.5g/L) for 5 min at 37°C to disassociate the cell monolayer from the cell culture dish. The trypsinized cells were then harvested and the whole cell suspension, consisting of the supernatant medium, the PBS-wash and the trypsinized cells, was spun down for 5 min at 1'000 rpm. All resulting supernatant was removed, and the remaining pellet was resuspended in 5 ml PBS. Another centrifugation step (5 min, 1'000 rpm) followed. This washing step with PBS was done twice. Going on with only the pellet, 1 ml of ethanol 70 %, pre cooled at -20°C, was added dropwise during slow vortexing to fix the cells. On this stage, cells had to be at least for 1 h on ice, but could have also been stored at 4°C for 2-3 weeks.

Before the samples were measured, the DNA had to be stained. Therefore, the cells, still in ethanol, were spun down for 6 min at 1'500 rpm. The supernatant was then decanted and the pellet was resuspended in 1 ml PBS. After another centrifugation step of now 8

min at 1’500 rpm and subsequent removing of the supernatant, each pellet was brought in suspension with 400 µl of the FACS solution. This mix was then immediately incubated in the dark for 30 min at 37°C. Afterwards, cells were ready to be measured. The measurements presented in this work, were performed by an experienced person on a Flow Cytometer CyAn ADP 9. If samples were not analyzed immediately, storage for a few days at 4°C was an adequate alternative.

4.8 SDS-PAGE and semi-dry western blot

Materials:

- MiniSpin centrifuge, Eppendorf
- Z216MK centrifuge (cooled), Hermle
- Bio-Rad protein assay, Bio-Rad
- 40% Acrylamide/Bis solution, 37.5:1, Serva
- PageRuler™ prestained protein ladder, Fermentas
- Immobilon-P transfer membrane, PVDF, Millipore
- Trans-Blot SD semi-dry transfer cell, Bio-Rad
- Extra thick blot paper, Bio-Rad
- Primary antibodies:
  - p97: serum was kindly provided by Hemmo Meyer, ETH Zürich, dilution: 1 in 2’000
  - Npl4: serum was kindly provided by Hemmo Meyer, ETH Zürich, dilution: 1 in 500
  - smc5: serum was kindly provided by Alan R. Lehmann, University of Sussex, United Kingdom, dilution: 1 in 1’000
  - mms21: NSE2 antibody, ab71976, Abcam, dilution: 1 in 500
- Secondary antibodies:
  - ECL Mouse IgG, HRP-linked whole antibody (from sheep), GE Healthcare
  - ECL Rabbit IgG, HRP-linked whole antibody (from donkey), GE Healthcare
- Chemiluminescent substrates:
  - SuperSignal West Femto Maximum Sensitivity Substrate, Thermo Scientific
  - SuperSignal West Dura Extended Duration Substrate, Thermo Scientific

- UptiLight HRP blot substrate, Uptima, Interchim

Method:

Western blot was performed to check the efficiency of the siRNA against the proteins of choice. Therefore, from each sample that was further processed according the "metaphase chromosome spread" protocol, 30 - 50 % of the cells were taken away for analysis. SDS-PAGE was then run to fraction the proteins by size. Once these proteins were transferred to a western blot membrane, specific antibodies allowed a quantitative analysis.

Samples:

To get the samples for gel electrophoresis and western blot, the "metaphase chromosome spread" protocol was carried out until the end of the first centrifugation step and all but approximately 300 µl of the supernatant was removed. After the pellet was resuspended by gently tapping, 30 - 50 % of the cell suspension was transferred in a small tube containing 500 µl PBS, and another spin down was performed for 5 min at 5'000 rpm in a MiniSpin centrifuge. To store the samples afterwards at -20°C, all supernatant had to be removed.

Whole cell extract:

It was important that the whole protocol for protein extraction was carried out on ice hence the samples gathered as describe above were, if frozen, thawed on ice. Then a centrifugation step for 5 min at 5'000 rpm at 4°C took place, and remaining supernatant was removed. To disrupt the cells, 50 µl lysis buffer was pipetted into the tubes, followed by hard vortexing and incubation on ice for 10 min. To separate the supernatant containing all the proteins from the debris, the samples were spun down at 4°C for 10 min at 16'000 g. The resulting supernatant was then transferred into a new tube, while the pellet was discarded. For equal loading on the gel, it was important to determine the protein concentration for each sample by the Bio-Rad protein assay.

To finally disrupt the disulfide bonds, to denature the proteins and give them an overall negative charge, loading buffer (10X) was added in an amount of 1/10 of the sample

volume (= µl of lysis buffer used). Properly mixed, the samples were boiled for 5 min at 90°C. After the hot samples cooled down at room temperature, a short flash spin down was needed to bring down the condensation. The samples prepared like this were stored at -20°C, if not loaded on a gel immediately.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE):
SDS-PAGE is a very widely used method to separate proteins and to determine their molecular weights. Preparation of stacking gels and separating gels was done according the Table 1.

Table 1. Protocol for SDS-PAGE gels

	Stacking gel (4.8%)	**7.5 %**	**10 %**	**12 %**	**13.5 %**	**15 %**
ddH_2O	3.15 ml	4.5 ml	4 ml	3.6 ml	3.3 ml	3 ml
Solution C	1.25 ml	---	---	---	---	---
Solution B	---	2 ml	2 ml	2 ml	2 ml	2 ml
40% acrylamid solution	0.6 ml	1.5 ml	2 ml	2.4 ml	2.7 ml	3 ml
10% ammonium persulfate	0.05 ml	0.08 ml	0.08 ml	0.08 ml	0.08 ml	0.08 ml
TEMED	0.005 ml	0.008 ml	0.008 ml	0.008 ml	0.008 ml	0.008 ml
	5 ml	8 ml	8 ml	8 ml	8 ml	8 ml

Semi-dry blot:
After an extra thick blot paper, well wetted with semi-dry transfer buffer, was placed in the semi-dry transfer cell, an activated transfer membrane was deposited thereupon. Further, the gel was carefully laid on the transfer membrane, before another extra thick blot paper, again wetted with semi-dry transfer buffer, completed the sandwich. Dependent on the size of the blotted protein and the acrylamid content of the gel, the blot was ran between 30 and 50 min with a constant voltage of 20 V.

After blotting, the transfer membrane was washed in PBS-T for several min. Subsequent incubation with the blocking solution lasted 1 h at room temperature. After the blocking step, the milk was decanted and the transfer membrane washed twice with PBS-T for 5 min.

Immunodetection:

First, the transfer membrane was incubated with the primary antibody for 90 min. Afterwards the membrane was washed three times with PBS-T, while each wash took 5 min. The same washing steps were repeated following the 30 min incubation with the secondary antibody. Now the membrane was ready for being visualized. Dependent on the cellular abundance of the protein to be determined, different chemiluminescent substrates had to be used.

5. Results

5.1 Mitotic index assay

The aim of this study was to characterize the influence of p97/VCP and its adaptors and potential substrates on the G2/M checkpoint. To observe the functional efficiency of the G2/M checkpoint, a mitotic index assay was established in U2OS cells (Figure 8).

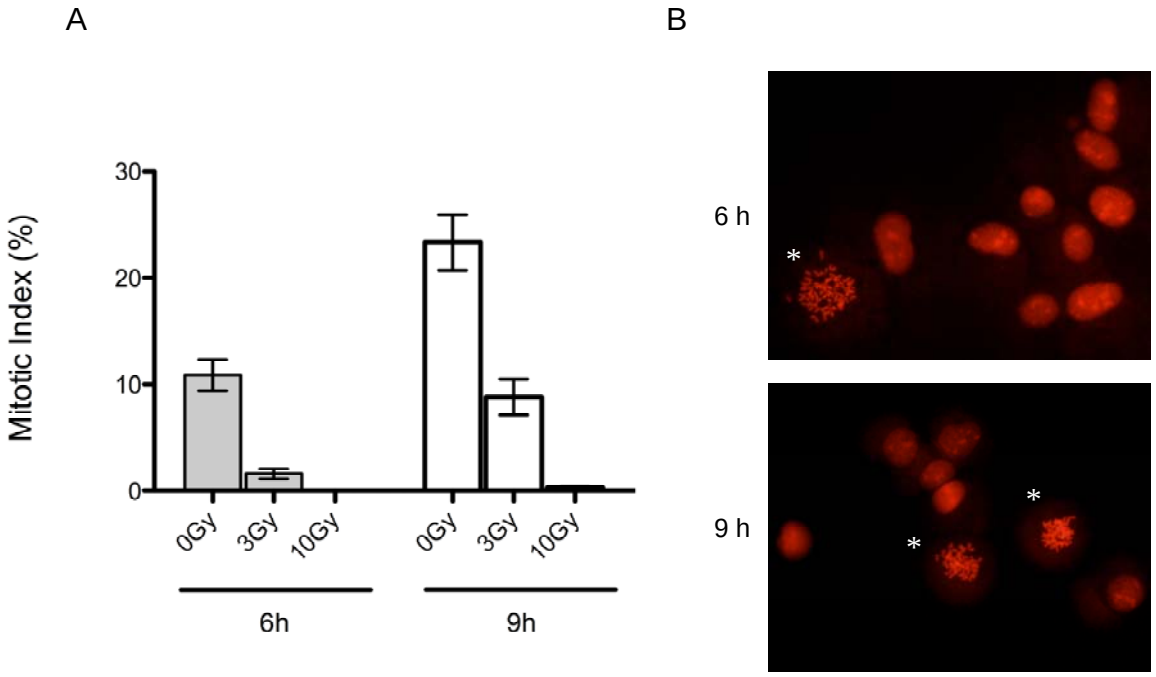


Figure 8: Activation of the G2/M checkpoint upon DNA damage measured by a mitotic index assay. The experiments were performed as outlined in Materials and Methods. **(A)** Percentage of non-silenced cells (ns) in mitosis at the time points 6h and 9h after treatment with 0, 3 and 10 Gy, respectively. The experiment was performed four times. Error bars are standard error of the mean (SEM). **(B)** Representative pictures of non-irradiated propidium iodide (PI) stained nuclei at the time points 6h and 9h, respectively. Asterisks mark mitotic nuclei.

Non-irradiated cells enter mitosis continuously, but upon irradiation, much less cells reach mitosis, which is most likely due to an activated G2/M checkpoint (Figure 8A). However, after 9 h, cells irradiated with 3 Gy start to recover, since the mitotic index ratio 0 Gy : 3 Gy becomes smaller, meaning that the cells were most probably able to

repair the damage and therefore were released from the G2/M checkpoint into mitosis. The DNA damage caused by 10 Gy appears to be so severe that cells are not able to recover within time periods used in this experiment. To quantify the amount of cells in mitosis versus interphase cells, the “metaphase chromosome spread” represents an easy and clear method (Figure 8B).

In summary, the mitotic index assay allows the observation of a checkpoint functionality, in all probability of the G2/M checkpoint, and this assay therefore was used for further experiments.

5.2 siRNA-mediated knockdown of p97/VCP, Npl4, smc5 and mms21 in asynchronized cells

Considering the mitotic index assay as a stable working frame, siRNA experiments targeting p97/VCP and its ubiquitin-related adaptor Npl4, as well as the potential substrates smc5 and mms21, were carried out.

A first overview reveals that the checkpoint functionality is not significantly affected in cells deficient for the indicated proteins (Figure 9). Even if the amount of cells entering mitosis varies between the distinct knockdowns, cells treated with ionizing irradiation always show a lower mitotic index. Whereas samples treated with 10 Gy are even more affected than those irradiated with 3 Gy. In summary, this first look indicates basically a functional checkpoint.

Consistent with previous studies (for details see Introduction), after 9 h, cells depleted of p97/VCP exhibit cell cycle progression problems, as even unchallenged cells seem to have difficulties to reach mitosis (Figure 9B). Due to the fact that p97/VCP is an essential protein, viable cells revealed a siRNA efficiency of about 80% (Figure 9C).

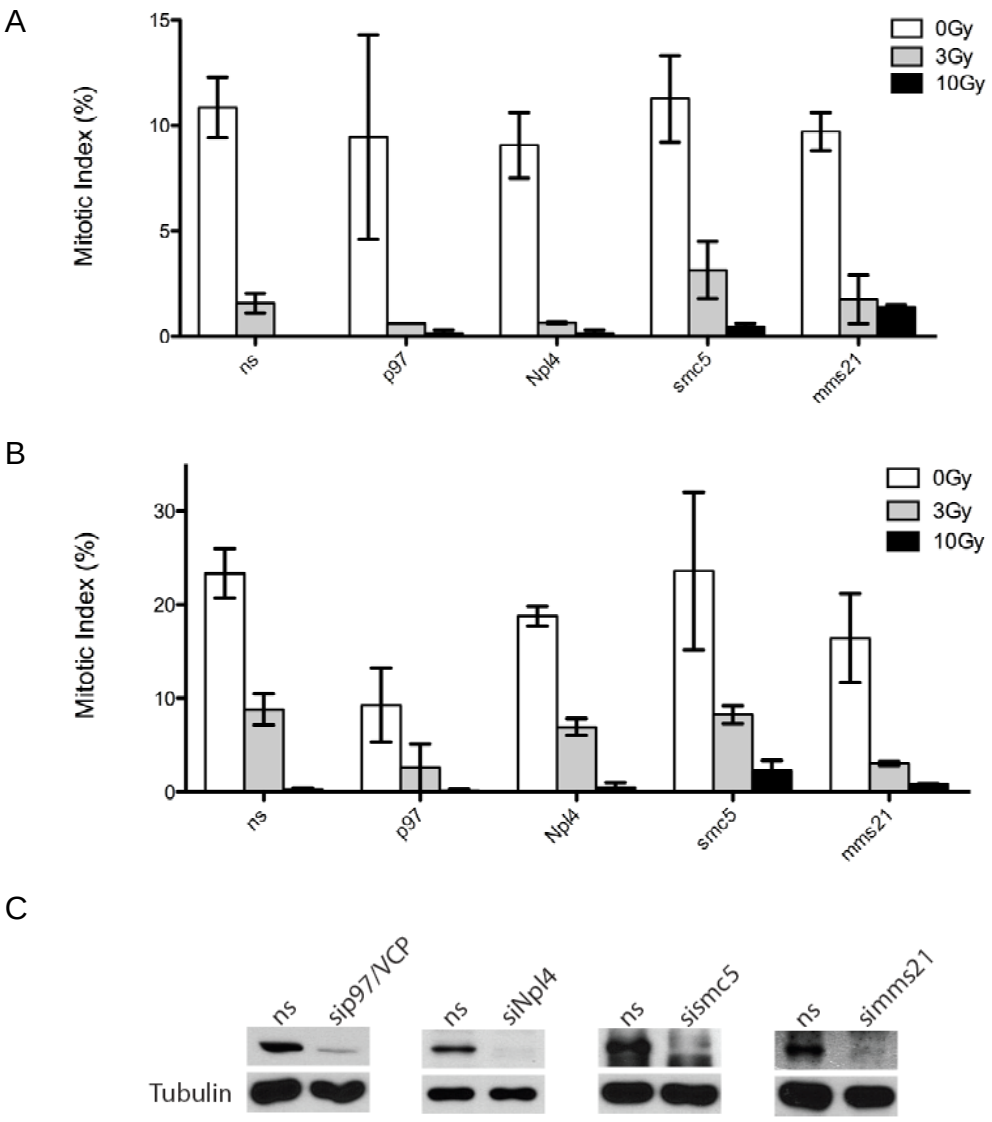


Figure 9: The G2/M checkpoint is not significantly affected by depletion of p97, its adaptor Npl4 or its potential substrates smc5 and mms21. The experiments were performed as outlined in Materials and Methods. **(A)** Percentage of cells in mitosis after 6 h. Cells were transfected with siRNA against the indicated proteins and exposed to ionizing radiation. Ns: non-silenced control **(B)** 9h time point. **(C)** Western blot analysis of siRNA-mediated knockdown efficiency. The experiment was performed in duplicates. Error bars are SEM.

Having a closer look, cells deficient for mms21 and irradiated with 10 Gy depict an obviously higher amount of mitotic cells after 6 h (Figure 9A). After 9 h, this observation disappears (Figure 9B). If these differences seen at the 6h time point really represent a leaking G2/M checkpoint, why are they then not visible in the samples treated with 3 Gy? And why do they vanish after 9 h? First, if the leakage is only very decent, it would get

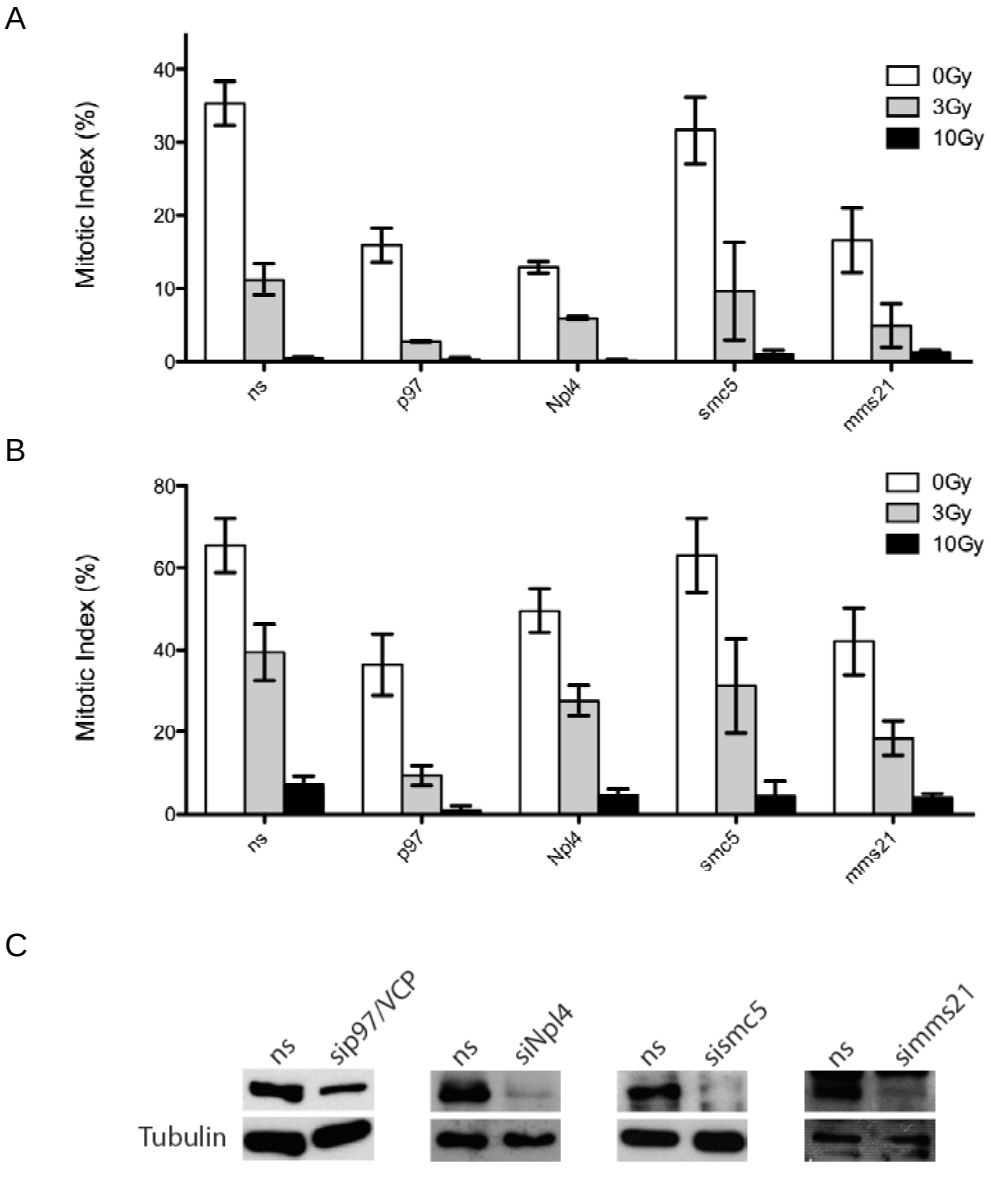
lost behind the physiological fluctuations of mitotic indices observed in cells irradiated with 3 Gy. Second, if the leakage is due to a delayed onset of the checkpoint, it would be reasonable that the observed difference is most prominent right at the time when the checkpoint becomes activated. Therefore, the more time passes by afterwards, the smaller the leakage becomes, relative to non-irradiated cells. However, the mitotic index assay determined by "metaphase chromosome spread" is not the most suitable method to properly detect such small differences, hence the observed differences have to be investigated by an additional experimental approach.

Even though the chosen time points allow to mainly detect the G2/M checkpoint, as cells way out of G2 are within 6 h or 9 h anyway not able to reach mitosis again. It can not be ruled out that other cell cycle checkpoints may affect the results, especially the intra-S phase checkpoint. To avoid this problem, cells were next synchronized on the G1/S boundary by a double thymidine block, released and irradiated at a time when they were assumed to be in G2.

5.3 siRNA-mediated knockdown of p97/VCP, Npl4, smc5 and mms21 in synchronized cells

By using a double thymidine block, it is possible to link the results much more precisely to the G2/M checkpoint. Additionally, the amount of cells detected in mitosis increases, since the cells are synchronized closer to mitosis and are not anymore randomly distributed throughout the cell cycle. At first view, the obtained results appear to be consistent with the experiment performed in asynchronized cells, since the functional efficiency of the G2/M checkpoint is also in synchronized cells not significantly affected (Figure 10A and B). Unfortunately, this conclusion was proven invalid, as a later FACS experiment showed that at the time point of irradiation (6h after release), cells were mostly in G1 and S phase and not in G2 as they were supposed to be (Figure 11A). Therefore, it is likely that additional checkpoint greatly influenced the observed results.

However, upon double thymidine block, a novel observation was evident. Strikingly, non-irradiated cells depleted of p97/VCP, Npl4 and mms21 show a decreased mitotic index at 6 h as well as at 9 h compared to non-silenced cells (Figure 10D). It is of course hard to assign the origin of this incident to a certain cell cycle event between the G1/S border and the M phase. Published data suggested, that CDC48$^{Ufd1/Npl4}$, the p97/VCP homologue, was implicated in DNA replication in *C. elegans* [178]. Moreover, mms21 is



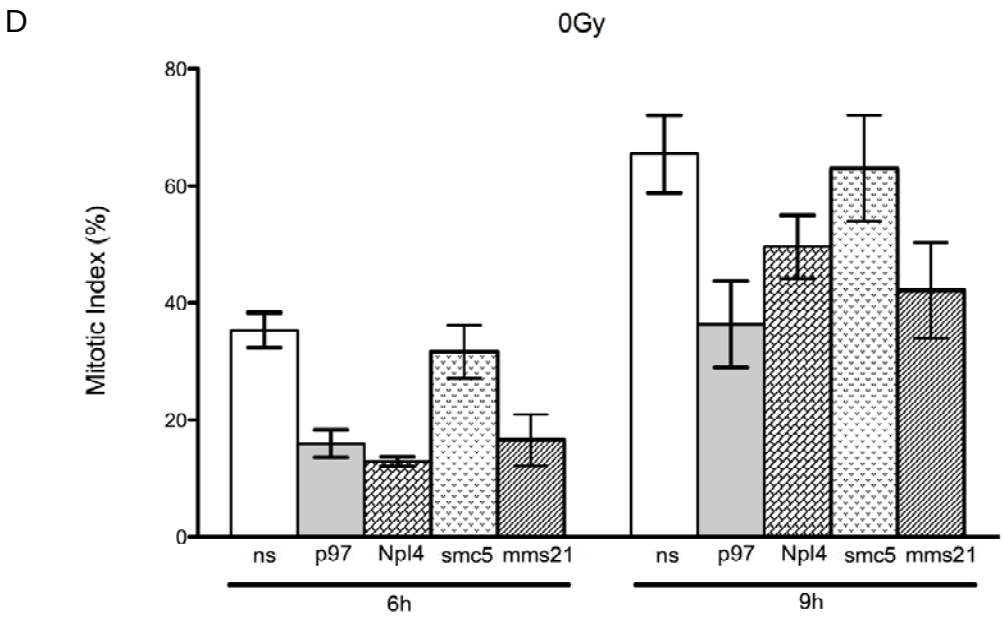


Figure 10: The G2/M checkpoint is not significantly affected by depletion of p97 and its adaptor or potential substrates. The experiments were performed as outlined in Materials and Methods. **(A)** Percentage of cells in mitosis after 6 h. Cells were transfected with siRNA against the indicated proteins and exposed to ionizing radiation. **(B)** 9h time point. **(C)** Western blot analysis of siRNA-mediated knockdown efficiency. **(D)** Summary of non-irradiated cells from (A) and (B). The experiment was performed in duplicates. Error bars are SEM.

known to facilitate homologous recombination [191]. In summary, the experimental data together with previous studies give a hint that upon double thymidine block, DNA replication and HR might be affected in human cells deficient for p97/VCP, Npl4 and mms21.

5.4 The two proteins, p97/VCP and Npl4 have an impact on DNA replication

In order to investigate the assumption that p97/VCP, its ubiquitin-related adaptor Npl4 and the E3 SUMO ligase mms21 are involved in DNA replication, a different experimental approach was established (mms21 remains to be tested). In detail, siRNA transfected cells were blocked at the G1/S boundary by using a double thymidine block. At the time of release and 6 h later, samples were collected and the DNA content was

measured by FACS analysis, whereby the efficiency of DNA synthesis became visible (Figure 11A).

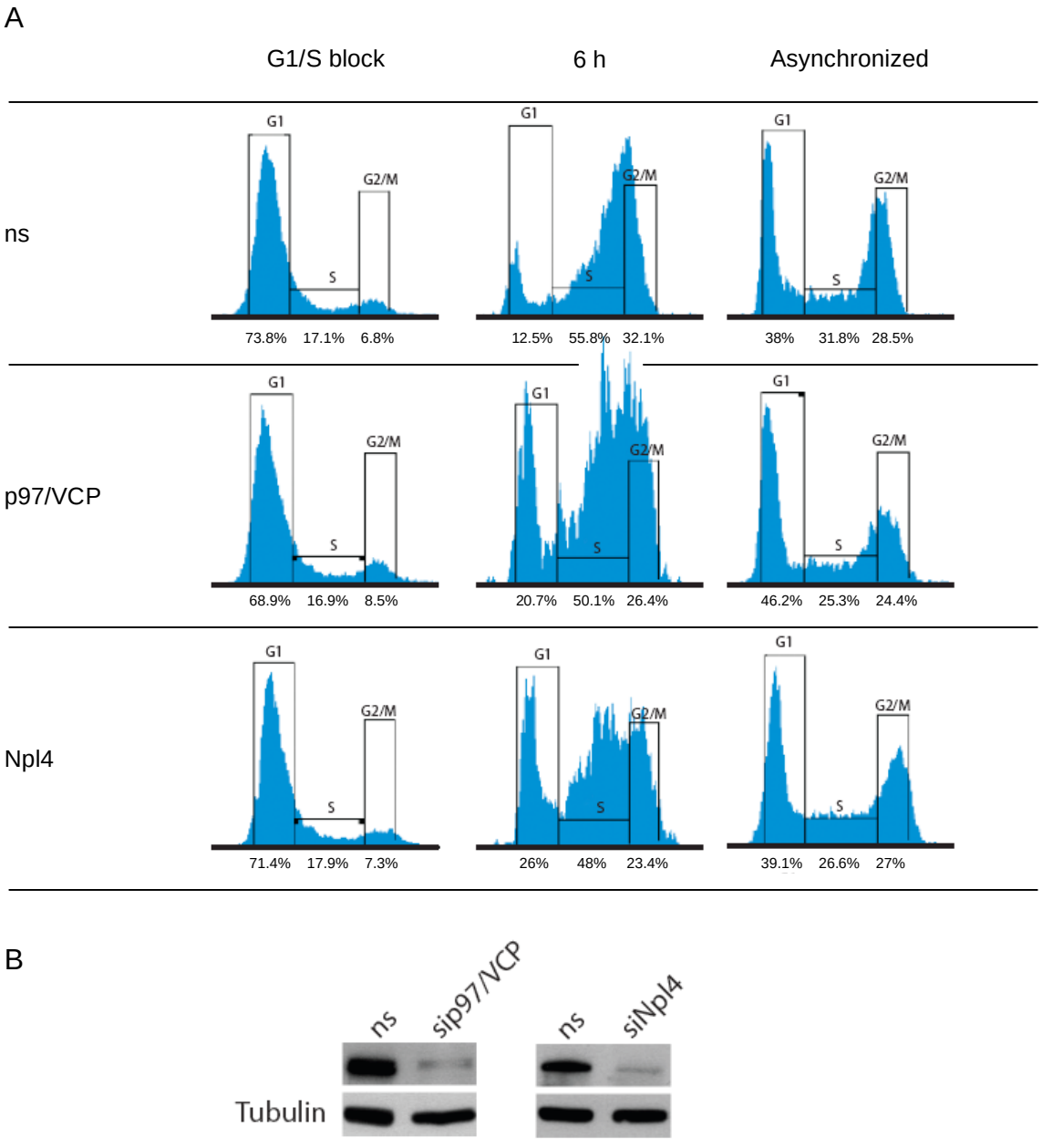


Figure 11: The S phase is delayed upon siRNA-mediated knockdown of p97/VCP and Npl4. The experiment was performed as outlined in Materials and Methods. **(A)** FACS cell cycle analysis of cells transfected with siRNA against the indicated proteins. Asynchronized cells were analyzed as controls. **(B)** Western blot analysis of siRNA-mediated knockdown efficiency. The experiment was performed only once.

Cell cycle analysis showed that non-silenced-cells enter and progress synchronously through the S phase, therefore these profiles were taken as reference values. Remarkably, both p97/VCP and Npl4 depleted cells depict a more diffuse profile during S phase. The initially sharp peak given by the synchronization becomes flattened, arguing for impaired DNA synthesis or a delayed and down-regulated initiation of DNA replication. But even more interesting is the fact, that after 6 h, in the same samples, a lot of cells are still arrested in G1. The most reasonable explanation therefore could be that these cells have a problem in the initiation of DNA replication, when p97/VCP and Npl4 are not present anymore.

6. Discussion

Initial results from our laboratory were the basis to closer investigate the role of p97/VCP in the DNA damage response pathway, most notably regarding cell cycle checkpoints. Therefore, the aim of the study was to characterize the influence of p97/VCP, its adaptor and potential substrates on the functional efficiency of the G2/M checkpoint. Furthermore, ongoing results led to the additional aim, to closer investigate the involvement of p97/VCP in S phase progression.

To set up a stable working frame, a mitotic index assay was first established. This assay showed a clear, dose-dependent (0 Gy, 3 Gy, 10 Gy) checkpoint activation, which was due to the chosen time points (6 and 9 h) most likely linked to the G2/M checkpoint. Experiments with asynchronized U2OS cells however, indicated that the G2/M checkpoint is not affected in cells depleted for p97/VCP, Npl4, smc5. Upon closer inspection, cells irradiated with 10 Gy and transfected with siRNA targeting mms21, depicted at 6 h a possible but decent leakage in the checkpoint. Since the observed difference is subtle, it is reasonable to assume, that in cells irradiated with 3 Gy the same leakage would get lost, as they exhibit a much higher physiological variation then the 10 Gy samples. As explanation, why this event is only observed at 6 h but not at 9 h, it can be hypothesized that the leakage is due to a delayed onset of the checkpoint. Therefore, once the checkpoint becomes activated, the observed initial leakage gets relatively smaller the more time passes.

To avoid interference with other cell cycle checkpoints, cells were synchronized and irradiated when they were assumed to be in G2. Unfortunately, a later FACS experiment showed that at the time point of irradiation (6h after release), cells were mostly in G1 and S phase and not in G2 as they were supposed to be (Figure 11A). Therefore, it is likely that additional checkpoints greatly influenced the observed results. Hence, no conclusions about the G2/M checkpoint should be drawn. However, these results exhibited valuable information, since non-irradiated cells depleted for p97/VCP, Npl4 and mms21 showed

after 6 h and 9h decreased mitotic indices if compared to control cells. This suggested an impaired cell cycle progression between the G1/S border and prior to mitosis (e.g. in S phase). According to published data [178], DNA replication appeared to be a potential origin of the problem. Subsequent FACS cell cycle analysis revealed that especially cells depleted for p97/VCP and Npl4 have problems to enter the S phase. 6 h after release from the double thymidine block, a lot of cells were still arrested in G1. Additionally, cells in S phase showed a delayed progression. Altogether, this mainly argues for an impaired initiation of DNA replication with eventually additional problems in DNA synthesis itself.

Many new interesting questions arise in view of this study. Regarding the observed problems during DNA replication, it remains to be confirmed, that p97/VCP and Npl4 are indeed involved in S phase initiation/progression, as the initial FACS experiment suggests (Figure 11A). If proven to be reproducible, the next question to answer will be, whether these two proteins play a role in a physiological context or only upon replication stress. Therefore, a new synchronization method might be used, since the double thymidine block is assumed to synchronize the cells on the G1/S border by stalled replication forks, what already represents replication stress. Synchronization during G1 would be preferable, to afterwards approach the S phase under physiological conditions. In this context, the urinary bladder carcinoma cell line T24 that arrests in G1 when confluent would be interesting to test. Once established, FACS cell cycle analysis of unchallenged and stressed cells as well as DNA synthesis assays (e.g. incorporation of radiolabelled thymidine or BrdU) would provide further insight. Concerning the G2/M checkpoint, the obtained insight from FACS cell cycle analysis in synchronized cells could be used in further experiments to precisely irradiate the cells in the G2 phase. Furthermore, it still remains to be solved, whether the slightly increased mitotic index noticed in asynchronized cells depleted for mms21 indeed represents leakage of the G2/M checkpoint. The advantage of the mitotic index assay determined by “metaphase chromosome spread” is, that during fluorescence microscopy the morphology of the mitotic figures as well as of interphase nuclei can be judged. However, due to the fact that only 300 cells were analyzed per sample, a second experimental approach has to be

chosen, to properly detect also small differences. The method of choice would be again FACS cell cycle analysis, whereby the cells are additionally incubated with fluorescent antibodies against a mitotic marker (e.g. phosphorylated histone H3-Ser10). Thus, it becomes possible to distinguish mitotic DNA from G2 DNA.

In conclusion the data presented in this thesis suggest that: first, the functional efficiency of the G2/M checkpoint is likely not regulated by p97/VCP, Npl4, smc5 and mms21; second, a prolonged G1 arrest and a delayed progression through the S phase, as observed in a preliminary FACS experiment, implicate a possible role of p97/VCP and Npl4 in initiating DNA replication.

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